Faculty of Resource Science and Technology

Rapid Amplification of cDNA End of Metallothionein Gene during Necrosis of Plant Cells from *Morinda citrifolia*

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Bachelor of Science with Honours
(Biotechnology Resource)
2008

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This Project is Submitted in Partial Fulfillment of the Requirements for the Degree of Bachelor of Sciences with Honors
(Resource Biotechnology)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2008

AKNOWLEDGEMENT

During the time when I was conducting my final year project, I had been supported and encouraged by many people. Here, I would like to express my deepest gratitude to all those that gave me precious help and support. Firstly, I would like to thank my supervisor, Dr. Hairul Azman Roslan, who gave me guidance, support and encouragement on every part of the project. He showed a great patience being the light on my way during the experiment and dedicated his time to analyse problem faced during the time of facing difficulty. I wish him a wonderful life. I am grateful when my beloved parents always stand by my side and encouraging me of not giving up during my desperate moment.

I would like to send my thanks to all the master students in Genetic Engineering Lab for giving me great help and advice during the my lab work. Working in lab with them was an invaluable moment. I am also thankful to lab assistants for their kind cooperation throughout the experimentation. Last but not least, I would also like to express my gratitude to my friends for their kind help, support and advice for accomplishing my project.

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ABSTRACT

The objective is to determine the presence of cysteine protease gene in the cDNA fragment being synthesized and determining the efficiency of the 3'RACE PCR approach for amplification of cDNA end. *Morinda citrifolia* is from the family Rubiaceae. It is the plant with great medicinal values. The property of this plant has not been characterized thoroughly in previous time. Cysteine protease play important role in plant growth and development in addition to protein degradation process in plant. Better understanding of the biochemical properties will be attained through the study of cysteine protease gene. The amino acids sequences of this enzyme govern the functional characteristic of this enzyme. Therefore, the termini of the cDNA encoding cysteine protease are amplified through Rapid Amplification of cDNA ends (RACE). This experiment was to amplify the 3' terminal of the cDNA nucleotide sequence. The RNA extraction was the preliminary step of the reverse transcription reaction to get the coding RNA fragment. First strand cDNA constructed was used for further step of 3' RACE by using gene specific primer and primers that enabled amplification 3'terminal nucleotides. The PCR products were gel extracted and sent for sequencing. Purified PCR products were cloned for further characterization and analysis of the isolated gene. The gene isolated was found to be similar to metallothionein-like gene and drought stress-induced gene when analysed through Blast.

Key words: RACE, cDNA, RNA, PCR

ABSTRAK

Objektif eksperimen ini adalah menentukan kehadiran gen cysteine protease dalam fragmen cDNA yang disintesiskan dan menentukan keberkesanan pendekatan 3'RACE yang diaplikasikan dalam mengamplifikasikan terminal cDNA. Morinda citrifolia berasal daripada famili Rubiaceae. Ia merupakan tumbuhan yang mempunyai nilai perubatan yang tinggi. Tumbuhan ini mempunyai banyak potensi yang belum ditemui lagi. Cysteine protease memainkan peranan yang penting dalam pertumbuhan dan perkembangan tumbuhan ditambah pula kepentingan enzim ini dalam prosess pemecahan protein. Pemahaman yang lebih mendalam tentang ciri-ciri biokimia tumbuhan akan diperolehi dalam kajian terhadap cysteine protease. Enzim ini mempunyai jujukan amino asid yang menentukan fungsinya. Oleh itu, bahagian terminal cDNA yang merekodkan protein cysteine protease diamplifikasikan melalui teknik khas yang dipanggil RACE. Eksperimen ini cuma mengamplifikasikan 3' nukleotida terminal cDNA itu. Pemencilan RNA merupakan teknik pertama sebelum langkah transkripsi berbalik untuk mendapatkan jujukan yang merekodkan gen berfungsi sahaja. cDNA yang disintesiskan itu digunakan dalam 3'RACE dengan menggunakan primer yang spesifik kepada gen sasaran dan primer yang membolehkan 3'nukleotida terminal cDNA itu diamplifikasikan. Hasil PCR akan dipencilkan dan kemudian untuk penjujukan. Hasil PCR yang tulen diklonkan untuk pengelasan and analisis gen itu. Gen fragmen itu didapati seiras dengan gen metallothionein dan gen berkaitan dengan tekanan kekurangan air apabila dianalysiskan dengan Blast.

Kata kunci: RACE, cDNA, RNA, PCR

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LIST OF ABBREVIATIONS

^{o}C	Degree Celsius
%	Percentage
bps	Base pairs
cDNA	Complementary DNA
CTAB	Hexadecyltrimethylammonium bomide
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphates
dT	Deoxythymidine
EDTA	Ethylenediaminetretraacetate
GSP	Gene specific primer
IPTG	Isopropyl-β-D-thiogalactoside
kb	Kilobase pairs
KCl	Potassium chloride
LB	Luria-Bertani Broth
LiCl	Lithium chloride
µl	Microlitre
µg	Microgram
$MgCl_2$	Magnesium Chloride
Min	Minutes
ml	Millilitre
mM	MilliMolars
MMuLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
mRNA	Messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
pmol	Picomole
Poly (A)	Poly adenosine
PVP	Polyvinylpyrrolidone
RACE	Rapid Amplifcation of cDNA Ends
RNase	Ribonuclease
RNasin	Ribonuclease Inhibitor
rpm	Revolution per time
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
RT PCR	Reverse transcription polymerase chain reaction
TAE	Tris-acetate EDTA electrophoresis buffer
U	Unit
UV	Ultraviolet
V	Volts
X-gal	5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside

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CHAPTER 1

INTRODUCTION

Morinda citrifolia plant is from the family Rubiaceae or its subfamily is Rubioideae (Nelson, 2006). It has been called Noni in Hawaii, and Mengkudu in Malaysia (Zin *et al.*, 2004). Most of the plant parts have medicinal values such as roots, flowers, seeds, leaves, barks and fruits (Wang *et al.*, 2002). Since *M. citrifolia* has a wide range of medicinal properties, this plant species is beneficial for used as research purposes. Most of the traditional and modern applications of this plant in vast number of ailments have yet to be scientifically proven. However, there were still several researches that had been done on the Noni fruit and root extracts that demonstrated the medicinal value of the plant. Besides, Noni has gained significance economic importance in the world today during recent years from the various types of health and cosmetic products which had been produced from leaves and fruits particularly (Nelson, 2006).

The study was conducted to have a more in depth understanding of Noni plant, by constructing cDNA ends encoding cysteine protease through Rapid Amplification of cDNA ends (RACE) method. There is still no published paper on the construction of cDNA encoding cysteine protease enzyme from *M. citrifolia*. Research had found that cysteine protease has multifunctional role. Cysteine protease also known as thiol proteases has important role during plant growth and development as well as programmed cell death (Grudkowska and Zagdanska, 2004). Since cysteine protease is very important in governing plant metabolism from stress respond, senescing, causing cell death, production of new proteins and even pathogenic resistant of plant, isolation of cysteine protease gene from *M. citrifolia* can be used for further research such as recombinant DNA technology. Complete sequence of cysteine

protease must be obtained through RACE before other research work related to cysteine protease can be performed.

RNA extraction was done since RNA only contains the coding regions of the gene encoding cysteine protease protein. Modified CTAB method from the method developed by Zeng and Yang (2002) was practised suitable for *M. citrifolia* RNA isolation (Tan and Roslan, 2006). The modifications being made include using chloroform to remove all the polysaccharides and secondary metabolites contaminants instead of using chloroform-isoamyl alcohol solution and the incubation period for precipitation of nucleic acid was four hours and not overnight incubation as in Zeng and Yang (2002) method.

RACE method is the common method applied to obtain complete sequence of specific gene by constructing the complete cDNA ends as traditional method applied such as the common process of RT-PCR usually result in only amplification of partial cDNA fragments. Besides, traditional method of obtaining gene specific sequence from the cDNA libraries through hybridization with radioisotope-labeled probes was unsuccessful and often includes intron sequence. RACE protocols can be used to generate specific gene in unlimited number of clones in short time compared to the traditional method that the researchers had to screen libraries with large number of clones which even caused more tedious and laborious work (Wang and Young, 2003).

The objectives of the study were to determine the presence of cysteine protease gene in the cDNA fragments constructed, determining the efficiency of the PCR approach for amplification of cDNA ends and extract of RNA from *M. citrifolia* leaf.

CHAPTER 2

LITERATURE REVIEW

2.1 *Morinda citriflolia*

2.1.1 Introduction of the plant

Morinda citriflolia plant is native to Southeast Asia (Indonesia) and Australia. The distributions of the plant are Eastern Polynesia such as Hawaii, Melanesia, and Western Polynesia such as Tonga, Indonesia, Australia and Malaysia. Noni plant can tolerate for an extremely wide range of environmental conditions including harsh environments such as basaltic lava flows. The plant also grows well in infertile, acidic, alkaline soils and relatively dry areas or low areas close to shorelines. It is the first plant colonizes harsh areas or lava flows. *M. citrifolia* plant can be found in brackish tide pools near coast, in limestone soils, coral atolls, coconut plantation, waste areas or native forest of 0-300m (Nelson, 2006).

The plant is generally an evergreen tree or shrub and will usually grow from 3 to 10 meter in heights when achieved maturity. The corolla of the flower is white in colour and lobed which is about 7-9mm long. The leaves grow in apposite direction and pinnately veined (Wang *et al.*, 2002). Different varieties of Noni plant exist due to different leaf morphology. The leaves can be rounded, elliptic or long and strap-like (McClatchey, 2002). Noni plant has fruit which is yellowish in colour and will become soft and fetid when ripe (Nelson, 2006) with some varieties are odorless and some varieties have strong butyric acid smell (McClatchey, 2002). The surface of the fruit is lumpy covered by polygonal-shaped sections (Wang *et al.*, 2002). The fruits contain seed which are triangular in shape, reddish brown and have air chamber (Wang *et al.*, 2002) and enable the seeds to retain viability even after

floating in water surface for months (Nelson, 2006). The root or bark part of the plant has been used by the Hawaiian as dye in painting on clothes (McClatchey, 2002).

Olden healers such as the Rotuman and Hawaiian gave treatment and prescription based on the *M. citrifolia* immature fruits or mature leaves as the main ingredients (McClatchey, 2002). According to the traditional treatments and scientific researches over the years, Noni is the plant with great botanical medication value. Malaysians used heated leaves of Noni plant to treat coughs, splenomegaly, nausea, fever and bacterial infection by placing them onto the infected area (Zaidan *et al.*, 2005).

2.1.2 Researches on *Morinda citrifolia*

According to Liu *et al.* (2001), two novel glycosides known as 6-*O*-(b-D-glucopyranosyl)-1-*O*-octanoyl-b-D-glucopyranose and asperusidic acid which was extracted from Noni fruit juice had chemopreventive effect. This indicated that the noni juice involved in the mechanism of tumor suppression. Noni juice had blocked AP-1 transactivation activity which had important role in tumorigenesis.

Zin *et al.* (2004) reported that chromatographic fractions for extraction from the root, fruit and leaf demonstrated considerably high antioxidative activity. Phenolic compounds with antioxidative activity were potent scavengers of free radicals and useful for prevention of arteriosclerosis, cancer, diabetis and arthritis. There was a possibility that the Noni associated arthritis and diabetis diseases relieving was caused by the existence of certain compounds such as scopoletin, nitric oxide, alkaloids and sterols in plant (Blanco *et al*., 2005).

Research had been done by Komaraiah *et al*. (2005) on suitability of certain elicitors, ultrasonication and sucrose feeding to enhance the accumulation of anthraquinones in *M. citrifolia* suspension culture. Anthraquinones were stored in root part of the plant species as

glycosides and aglycones. The use of root in herbal preparation for various disease treatments showed the importance of the anthraquinone compounds production in *M. citrifolia*. Polyunsaturated fatty acids such as linoleic acid, α-linolenic acid, arachidonic acid, methyl jasmonate, salicylate, and sodium nitroprusside had shown to be effective to stimulate anthraquinones production in *M. citrifolia*. The combination of sucrose and methyl jasmonate gave even higher yield to anthraquinone production which was synergistic. In addition, treatment of the suspension culture with ultrasonocation had increased the accumulation of anthraquinones by four-fold. The anthraquinones in fruit was not enough in terms of quantity to cause liver tissue damage (West *et al.*, 2006).

In addition, study done by Zaidan *et al.* (2005) showed that *M. citrifolia* leaves had antibacterial activity towards gram positive *Staphylococcus aureus* and Methicillin Resistant Staphylococcus (MRSA) but not to *Escherichia coli* and *Klebsiella pneumoniae*. Acute toxicity test on the Noni fruit crude extracts was found to be greater than the minimum criteria for nontoxic status.

2.2 Leaf senescence

Leaf senescence is the condition occurred when there are changes in cell structure, metabolism, and gene expression (Gan and Amasino, 1997). Senescence that occurs in higher plants gone through programmed cell death (Lim *et al.*, 2003). Examples of enzymes involved in senescence are cysteine protease (Beyene *et al.*, 2006), metallothionein (Buchanan-Wolleston, 1994), ubiquitin specific protease, kinases (Navapbour *et al.*, 2007) and lipases (Ryu and Wong, 1995 cited in Gan and Amasino, 1997). There are two types of senescence which are replicative senescence and post-mitotic senescence. Replicative senescence is the loss of plant capacity for further cell division during aging while post-mitotic senescence

refers to a degenerative process that occurs after cell differentiation and which leads to cell death (Lim *et al.*, 2003).

Leaf senescence is regulated by environmental factors such as extremes temperature, drought stress, ozone, nutrient deficiency, pathogen infection, wounding and shading as well as internal factors such as age, reproductive development and levels of phytohormone. The most significant change in the cell which cause symptom of leaf yellowing is breakdown of chloroplast whereby chlorophyll and macromolecules catabolism occurred replacing carbon assimilation caused the photosynthetic rate drops below certain threshold. These changes are caused by changes in gene expression including nuclear gene expression. Nutrients released from the macromolecules are translocated to other young leaves, developing plant organs, reproductive organs or storage tissues. A group of senescence-associated genes that are up-regulated during senescence were found and the regulation of the genes may be involving multiple pathways that form a regulatory network (Gan and Amasino, 1997).

2.3 Cysteine protease

2.3.1 Roles of cysteine protease

Vierstra (1989) reported that cysteine protease is important for protein degradation during seed germination in seed storage protein. Hydrolysis of the protein in seed storage will provide amino acids for new protein synthesis in the growing seedling. Callis (1995) found that selective degradation of protein in non-senescing organ in turn will serve to reduce the toxic effects of inactivated, denatured, unassembled and abnormal proteins. Furthermore, cysteine protease is involved in senescence and programmed cell death. Programmed cell death is executed by metacaspases during plant embryogenesis and depended upon its

cysteine-dependent arginine-specific proteolytic activity (Bozhkov *et al.*, 2005). Programmed cell death is involved in the elimination of proembryogenentic masses and embryo suspensor.

Cysteine protease participates in both anabolic and catabolic processes. Cysteine protease is synthesized at polyribosomes as large precursor that consists of short N-terminal and much longer C-terminal propeptides. It is produced in necrosis leaves that contain dead cells and in seed undergo germination. The plant cysteine proteases are located in vacuoles and in the cell wall. It is endopeptidase with a cysteine residue in their active site. Iodoacetate, iodoacetamide, and E64 are the active site inhibitors used to identify cysteine protease. Activation of the enzymes by thiol compounds is also used to identify this enzyme (Grudkowska and Zagdanska, 2004). Most of the cysteine protease that had been studied showed acidic pH optima. Most plant cysteine protease belongs to the papain-like (C1) and legumain (C3) family (Ghosh *et al.*, 2007). Five other members of the cysteine proteases that have been found are caspases (family C14), calpains, the calcium-dependent proteases (family C2), ubiquitin C-terminal hydrolases (family C12) and ubiquitin-specific proteinases (Vierstra, 2003 cited in Grudkowska and Zagdanska, 2004).

According to Grudkowska and Zagdanska (2004), cysteine protease has multifunctional roles with its activity in mature non-senescing organs up to 30% of total proteolytic activity. However, different internal and external stimuli will cause the enzyme's activity rise up to 90% of the total proteolytic activity. Proteolysis in plant by cysteine protease is a highly regulated process. The ability of the plant to modulate protein levels is necessary for the plant to adapt to changes in environment and to internal developmental signals. Besides, the enzymes also concern with the storage protein deposited in the cotyledon mesophyll of dicotyledonous plants and cereals endosperm tissue. Water deficit stress had

caused changes in proteolytic activity of ten genotypes of spring wheat of *Triticum aestivum* L. (Folinova *et al.*, 2000).

2.3.2 Cysteine protease related researches

Cysteine protease has been found in plants, animals, bacteria and eukaryotic microorganisms. Early resistant responses of potato towards *Phytophthora infestans* had enabled the isolation of cDNA clones encoding cysteine protease from this plant. Both the nucleotide and deduced amino acid sequences showed high homology to tomato cysteine protease and cathepsins in animals particularly cathepsin K. Comparison of the cysteine protease domains in potato and tomato cysteine protease as well as rice oryzain α uncovered that amino acids important for structural and functional activity of cysteine protease were conserved among them (Avrova *et al.*, 1999).

Harrak *et al.* (2001) had done research on elucidating the nature of the protein (TDI-65) resulted from drought-stresses tomato (*Lycopersicon esculentum*) plants. A cDNA clone (tdi-65) was isolated which had high homology with known cysteine protease such as actinidin and papain. Amino acids important for the structure and function of the cysteine proteases are conserved in tdi-65 protein. The size of the tdi-65 protein was approximately the same as TDI-65. The protein was found to be located in nuclei, chloroplasts and some leaf cytoplasmic regions. Results from Northern blot analysis showed that tdi-65 mRNA is 10-fold higher in drought-stressed plants when compared to control and rewatered plants. The result suggested the role of tdi-65 in protein degradation localized in chloroplast and nuclei during drought stress condition.

According to Kusaka *et al.* (2004), cysteine protease is the important component in causing the changes in morphological character of isolated nuclei in plants by using oat

(*Avena sativa* L.) for the research. In this experiment, cysteine protease was shown to be involved in executive phase of oat apotosis by inducing DNA laddering during programmed cell death. Moreover, cysteine protease was indicated to be essential for nuclear DNA fragmentation in the presence of nuclease.

According to Asp *et al.* (2004), three groups of genes comprise of two full length and three partial cysteine protease genes from white clover (*Trifolium repens*) were expressed in nodules, primarily the senescence zone but not in roots, leaves, petioles and internodes. The identified cysteine protease genes from *Trifolium repens* were shown to be highly homologous to cysteine protease gene from *Astragalus sinicus*, *Alnus glutinosa* and *Medicago truncatula*. On the other hands, the expression patterns of cysteine protease in sweet potato (*Ipomoea batatas*) storage roots have been reported by Dong *et al.* (2005). The expression of cysteine protease was found to be highest in storage roots compared to sprouted roots, green leaves and flowers.

Sheokand *et al.* (2005) demonstrated the important function and localization of cysteine protease (Cyp15a) in plant by modifying the gene encoding cysteine protease resulted in the production of non-functional protein. The transgenic lines being used is *Medicago truncatula* (R108-1). There was strong expression of promoter Cyp15 in cotyledonary leaves, senescent leaves and root nodules and its increased expression in the present of 0.6M mannitol and increased concentration of NaCl which indicated the role of Cyp15 as stress-inducible gene. Suppression of cysteine protease expression demonstrated no regeneration of plant and no seed germination occurred. Besides, plant range from slow growing mode and fatal phenotype to leafy and late senenscing kinds which demonstrated function of Cyp15a cysteine protease in seed germination and stress adaptation of plant.

Cysteine protease was induced to be expressed in rice (*Oryza sativa* L.) infected with blast fungus, *Magnaporthe grisea*. Oryzain α-A induced gene was not involved in rice resistant towards blast fungus but may be a factor for pathogen-induced necrosis. Furthermore, expression of oryzain α-A can be induced through wounding, ultraviolet radiation and treatment with salicylic acid (Fu *et al.*, 2007).

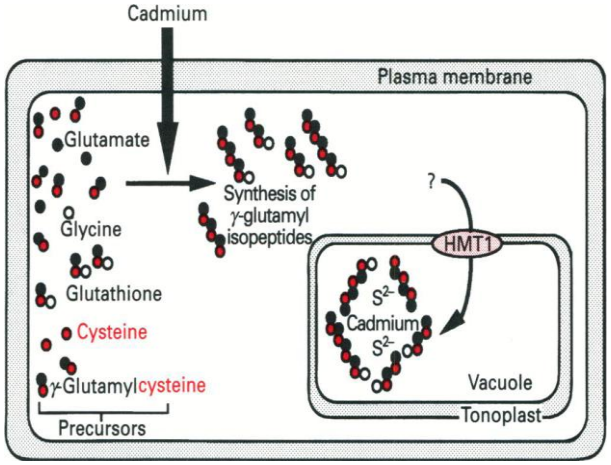
According to Yamauchi (2007), gibberellins (GA) and brassinosteroids (BR) overcome the repression of abscisic acid (ABA) on the expression of germination-induced cysteine protease genes (GICPs) in cotyledons of common bean seeds. Result showed that the mRNAs levels for all the cDNAs except two of the cDNAs code for papain-like proteases could not be detected in cotyledons that had been treated with 10μM abscisic acids. The mRNA levels increased after treatment with gibberellins or brassinosteroids. Result from the experiment suggested that gibberellins and brassinosteroids are essential for the expression of germination-induced cysteine protease genes in bean seeds or cereal seeds.

2.4 Metallothionein (Cysteine rich protein)

Metallothionein is a low molecular weight cysteine-rich protein. Metallothionein in plants and animals has metal binding properties within the cells with the potential function in metabolism and homeostasis of different metal ions. The metal binding properties play essential role in homeostasis of metal ions such as zinc and copper ions and detoxification of metal ions such as cadmium, zinc and copper ions (Jin *et al.*, 2006). There was also report claim that the binding with metal ion protected protein from proteolytic degradation. Presently, there are 3 classes of metallothionein-like proteins found in terms of Cys residues locations which are class I, II and III and Class II metallothionein can be further categorized into four types which are type 1, 2, 3 and Ec (early cystein labeled) protein according to the amino acid sequence in

cysteine domains (Cobbett and Goldbrough, 2002). Different plants with metallothionein proteins from different gene family expressed in different organs. For example, metallothionein expressed in soya bean roots and abundantly in leaves while in pea plants, the metallothionein was abundant during growth of the root organs in hydroponic culture (Robinson *et al*., 1993).

According to Robinson *et al.* (1993), there had been different theories regarding the biosynthesis of Class III metallothionein. However, the theories were mainly on the synthesis of Class III metallothionein from glutathione. Structural similarities between glutathione and Class III metallothionein had gave rise to the possibility that Class III metallothionein synthesized from glutathione. Synthesis of the metallothionein from glutathione by enzyme γ-glutamylcysteine dipeptidyl transpeptidase had been reported in *Silence cucubalus* cell suspension. This biosynthesis mechanism needed two glutathione molecules or one glutathione with previously produced metallothionein. There was also theory regarding the inhibition of glutathione biosynthesis by cadmium ions in maize caused accumulation of γ-glutamylcysteine which led to the synthesis of Class III metallothionein. Another alternative pathway for the biosynthesis was the polymerization of γ-glutamylcysteine by the transfer of γ-glutamylcysteine from glutathione to another $(\gamma\text{-glutamylcysteine})_n$ to produce $(\text{glutamylcysteine})_{n+1}$ and glycine. Figure below shows the illustration of this pathway.



Adapted from Robinson *et al.* (1993). Class III metallothionein shown in the figure above contain multiple polypeptide molecules and some of the complexes contain inorganic S^{2-} in the metal core. HMTl gene located in the vacouler membrane which is required for accumulation of metallothionein in vacuoles. Red circles, cysteine residues; black circles, glutamate residues; white circles, glycine residues

2.5 Drought stress-induced gene

The decreased content of water in the soil had caused changes in gene expression patterns which increased the rate of transcription of a specific gene in respond to drought stress (Bray, 2002). Drought stress is the condition when the water potential around the cell lowered and there is reduced cell growth (Cramer and Bowman, 1994). The gene expressed during water deficit condition might function to promote the survival of the plant (Bray, 2002). Examples of drought-stress induced gene are cysteine protease (Harrak *et al*., 2001), metallothionein-like protein (Jin *et al.*, 2006) and glutathione-*S*-transferase (Reymond *et al.*, 2000). The gene expression was induced by a complex series of signal transduction events not fully defined. Abscisic acid is one of the signal molecules that might confer adaptation function. There are 11 functional categories of genes induced by drought stress such as cell defence, cell death, ageing, protein metabolism and ionic homeostasis (Bray, 2002). Other categories of drought stress induced gene have protein of unknown function. Water deficit condition has decreased

the availability of the mineral ions in soil (Alam, 1994). The plant adapt to the drought stress by a few modifications in its structures and processes such as stomatal response and osmotic adjustments (Pugnaire *et al.*, 1994).

There are three mechanisms of drought resistance which are drought avoidance, drought escape and drought tolerance depending on the plant response to drought (Mitra, 2001). Plant showed drought avoidance when it is able to maintain high water potential in water depletion soil (Mitra, 2001) or when the plant restrict its activity until water content return to normal (Pugnaire *et al.*, 1994). Drought escape is demonstrated when the plant complete its life cycle before serious water deficit condition of the soil occur whereas drought tolerance is the ability of the plant to survive in water depletion soil by maintaining low water potential in the tissues (Mitra, 2001) through decreasing osmotic potential (Pugnaire *et al*., 1994).

2.6 Rapid Amplification of cDNA ends (RACE)

Rapid Amplification of cDNA Ends (RACE) encoding cysteine protease is a type of polymerase chain reaction (PCR) based technique which was developed to enable the cloning of full-length cDNA 5'- and 3'-ends after obtaining partial cDNA fragment (Schaefer, 1995). There are two general RACE strategies that are 3'RACE which amplifies 3' cDNA ends and 5'RACE which amplifies 5'cDNA end sequences (Wang and Young, 2003). This method has been used widely to determine the 5' and 3' terminal nucleotide sequences of the genes (Shi and Jarvis, 2006).

First round of the RACE would usually produced a high background of nonspecific RT-PCR products which necessitates the second round of RACE with another set of nested gene specific primers to enable the construction of cDNA encoding cysteine protease with